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PRINCIPAL INVESTIGATOR: Sylvia M. Kiertscher, Ph.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, California 90095-1405

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Sylvia M. Kiertscher, Ph.D.

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University of California
Los Angeles, California 90095-1405

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13. ABSTRACT *(Maximum 200 words)*

Dendritic cells (DC) are an integral part of the immune system's response to cancer. A number of clinical trials have been initiated to use these cells in the treatment of melanoma, prostate cancer, and lymphoma. In preparation for treating breast cancer patients with DC, this proposal examines a fundamental issue that needs to be resolved before proceeding with this exciting new therapy. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC is a more efficient and effective way of stimulating T cell responses than current HLA-restricted peptide-based methods. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In the past year we have begun recruiting patients for *in vitro* studies of antigen-presentation and have optimized testing procedures to evaluate anti-tumor cytotoxicity and tumor antigen-specific cytokine release by T cells. In addition, we have devised a strategy for expressing the Her-2 gene in DC and evaluated methods for preparing tumor lysate and whole tumor antigen. This progress is consistent with the proposal's Statement of Work, and leaves us well-positioned to achieve the next goals of the proposal.

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FOREWORD

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S. M. Kwiatkowski 7-28-99

PI - Signature Date

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INTRODUCTION

Dendritic cells (DC) are an integral part of the immune systems' response to cancer. When loaded with tumor antigens, DC have great value as immunotherapeutic agents. However, the method for arming the DC with antigens which results in the most effective immune response has yet to be defined. We theorized that the broadest possible mix of tumor antigens might provide the best material for stimulating an effective immune response. We hypothesized that the processing and presentation of multiple tumor antigen epitopes by DC would be the most efficient and effective way of stimulating T cell responses. The goal of this proposal is to develop practical methods by which immune cells from patients with breast cancer can be used to promote effective anti-tumor responses. In this study we will compare multiple methods of arming DC with tumor antigens including: 1) purified immunodominant peptides which are specific for a single antigen and a single Class I MHC molecule, 2) transduced cDNA encoding for a single tumor antigen which will allow the recipient DC to intrinsically process and present all possible antigenic peptides (immunodominant and sub-dominant) within the context of all available MHC molecules, and 3) extracts from autologous whole tumor cells which will provide a broad mix of tumor antigens (both defined and undefined antigens) for processing and presentation. The information obtained from this study will further our understanding of the interactions between DC and T cells which lead to the generation of tumor-antigen-specific responses. This understanding will be valuable in the development of immunotherapeutic treatments for breast cancer.

ANNUAL SUMMARY

For the year 7-1-98 through 6-30-99 (proposal months 0-12), we are pleased to report progress on Tasks 1-3 in the proposal Statement of Work. This progress is composed of advances in three main research areas: patient recruitment, Her-2 antigen preparation, and development of assays to assess anti-tumor reactivity.

Task 1. To identify HLA-A2$^+$ breast cancer patients whose tumors do and do not overexpress Her-2/neu. (Scheduled for months 1-36).

Patient Recruitment

The UCLA IRB has approved the recruitment flier and informed consent forms for the proposal. The process of patient recruitment is underway. Based on the progress made in optimizing study assays and materials in this past year, it is expected that the enrollment of patients in *ex vivo* studies will occur in the upcoming grant year. Our collaborator (Mark Pegram, M.D., Division of Hematology and Oncology, UCLA School of Medicine) routinely screens patient tumors for Her-2 expression, so many prospective patients already know their Her-2 status and will only require HLA-typing prior to enrollment.

Evaluation of Patient Dendritic Cells

The proposal currently calls for patient dendritic cells (DC) to be evaluated for antigen-presenting cell phenotype and T cell stimulatory activity. Recent research has established the importance of IL-12 production by DC in their interaction with T cells, and in the propagation of an effective anti-tumor Th1 T cell response. Exposure to tumor-derived substances can inhibit the DC's ability to produce IL-12. Therefore, in addition to evaluating DC phenotype and stimulatory function, we will also test patient's DC for their ability to produce IL-12. It is possible that exposure to tumor *in vivo* will affect this ability, but that exposure to cytokines *ex vivo* may overcome this effect. If patient's DC lack the ability to produce IL-12, the addition of exogenous IL-12 to *in vitro* assays, (and to eventual *in vivo* therapies with DC), may be required.

Task 2. To obtain peripheral blood and tumor specimens from these patients and use them to generate DC, isolate T cells and produce tumor cell lysates. (Scheduled for months 2-40).

Tumor Lysate Preparation and Use:

Preparation of tumor lysate from patient's tumor samples is an important prerequisite for the assays in Task 3 and 4. One concern in the preparation of these lysates is that tumors also contain tumor infiltrating lymphocytes. Because the lysates will be used to arm DC, which are potent antigen-presenting cells, the presence of lymphocyte antigens may result in the generation of anti-lymphocyte autoimmune responses. In the past year, we have tested methods to remove lymphocytes from the tumor digest cells before the preparation of the lysates. As shown in Figure 1, we found that treatment of the cells with antibody directed against the pan-leukocyte antigen CD45 and immunomagnetic beads effectively removed the lymphocytes. The left panels show the tumor

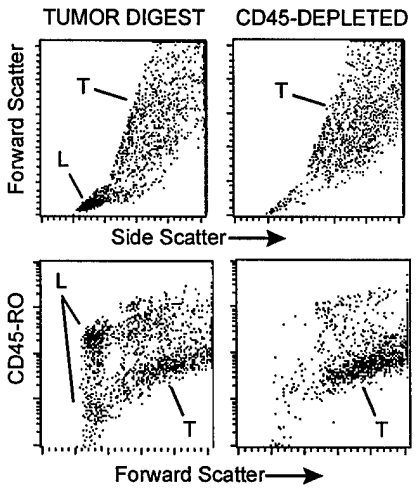

Figure 1. Treatment of tumor digest cells with anti-CD45 antibody removes tumor infiltrating lymphocytes.

digest cells before treatment. They are composed of 43% lymphocytes (indicated by L) and 56.9% tumor cells (indicated by T). After treatment with anti-CD45, the cells are 3.6% lymphocytes and 96.4% tumor cells (right panels). The bottom panels show that both the $CD45RO^+$ and $CD45RA^+$ lymphocyte populations are removed. We also determined the best method of disrupting the tumor cells to produce antigens in a form usable by DC. We compared the use of multiple freeze-thaw cycles to sonication, and found that sonication disrupts all of the tumor cells into small cell fragments, resulting in higher yields of released proteins (283 $\mu g/10^6$ cells, compared to 192 $\mu g/10^6$ cells for freeze-thaw). Finally, we determined the most effective way to arm DC with lysate by comparing the use of liposome-loading methods to lysate alone. Utilizing FITC-ovalbumin as an model antigen, we used fluorescence analysis to follow the amount of antigen taken up by the DC. We found that liposomes reduced the amount of time required to load DC with antigen (20 min. vs. several hours), but that there were major disadvantages. These included loss of DC viability, changes in DC cell surface marker expression, and difficulty in reproducibly achieving the correct protein:liposome ratios. Consequently, we have decided to use the lysate directly on the DC, requiring a slightly longer antigen exposure time. Based on this series of experiments, we have developed the following protocol for tumor lysate preparation and use: (1) tumor samples will be treated with enzymes to produce a single cell suspension, (2) lymphocytes will be removed with anti-CD45, (3) the remaining tumor cells will be disrupted by sonication, (4) the protein concentration of the lysate will be measured, and (5) the lysate will be frozen in aliquots for future use. When needed for the assays, the lysates will be thawed and used to arm DC with antigens by co-incubation for 2-4 hours.

Task 3. To determine the frequency of Her-2-specific T cells generated using the three different antigen-arming methods with a modified limiting dilution procedure. (Scheduled for months 2-40).

Her-2 Gene Expression in DC

One of the antigen-arming methods to be used in this task is transduction of DC with the Her-2 cDNA. The use of adenoviral vectors is still the most effective method for expressing transgenes in DC, and with the assistance of Dr. Lisa Butterfield, Division of Surgical Oncology, UCLA (an expert in the use of adenoviral vectors in DC), we have developed a strategy for Her-2 gene expression using these vectors. The Her-2 cDNA is currently in a retroviral expression vector, and we are in the process of excising it from this vector and inserting it in the adenoviral vector. The restriction sites at the ends of the Her-2 gene do not correspond to the sites in the adenoviral expression vector insert region, so it is necessary to use blunt end ligation or primer extension to insert the gene in the vector. Both approaches are being used to maximize our chances of early success. The use of alternative helper-dependent viral systems to express transgenes in DC is under development. The advantage to the helper-dependent virus is its lack of expression of viral genes, and the corresponding reduction in the possibility that anti-adenoviral immune responses will interfere with DC function. However, these virus are difficult to produce and are not currently available in sufficient quantities to use. They will be used in later studies if it becomes feasible. In related DC research, we have determined that less "mature" DC are easier to transduce, but are also more sensitive to virally-mediated down-regulation of cell surface molecules. Intermediately mature Day 6 DC appear to retain their ability to be transduced, while being relatively resistant to the deleterious effects of virus on phenotype. For this proposal we will use Day 6 DC, rather than the mature Day 7 DC, to transduce with the Her-2 gene. This will also allow the cells 24 hours to upregulate Her-2 gene expression prior to use in the generation of tumor-specific T cells.

Additional Antigen Sources

The proposal was originally designed to test antigens from three sources (peptide, tumor lysate, and gene expression). Recent research has suggested that apoptotic cell bodies derived from tumor cells could be a potent source of tumor antigens. These bodies are membrane vesicles which are shed by tumor cells undergoing apoptosis. They express both MHC and tumor antigens on their cell surface, are preferentially taken up by DC for antigen processing and presentation, and have been used to promote anti-tumor activity in mice. When the size of the tumor sample allows, we will induce apoptosis by UV exposure, and use the resulting apoptotic cell bodies to arm DC.

One limitation of our proposal is that tumor specimens may not always be available in sufficient quantities to perform all of the planned experiments. An alternative method is to use the whole p185 Her-2 protein to arm DC. We have investigated the extraction of Her-2 protein from the breast cancer cell line SKBr3 as source of Her-2 antigens, and will use it when tumor samples are small or lacking. These additional sources of tumor antigen will allow a more complete evaluation of DC tumor antigen presentation than the current protocol, and will eventually enable us to treat a larger patient population.

Assay Development

Assays to monitor the generation of Her-2-specific T cell responses are essential to both this proposal (used in Tasks 3 and 4), and future clinical trials. In preparation for these studies, we have evaluated a new assay for the assessment of tumor cytotoxicity. The alamar blue assay uses a non-toxic metabolic indicator of viable cells that fluoresces upon mitochondrial reduction. Viable cells exhibit a measurable level of this activity, and reductions in this level indicate cell death. As shown in Figure 2, this assay measures cytotoxicity as effectively as the ^{51}Cr-release assay, and is, in fact, more sensitive at lower Effector:Target cell ratios. One of the drawbacks to the ^{51}Cr-release assay is that it relies on the ability of the tumor cells to take up and retain chromium before lysis by the effector cells. Cells which do not take up chromium well (such as fresh tumor specimens) are difficult to evaluate by this method. Because the ultimate test of our DC-stimulated T cells is their ability to lyse autologous tumor, it is important that we can measure this activity accurately. In contrast to the ^{51}Cr-release assay, the alamar blue assay can be used on all cell types. Another advantage is that alamar blue is non-toxic, and T cells can be recovered and grown from the assay wells. If one T cell population performs exceptionally well in the assay, it can be collected and expanded for further studies. A second critical assay for the evaluation of the generation of tumor-specific T cell responses is the ELISpot assay, which measures the frequency of cytokine-producing cells. In the past year, we have developed and standardized optimal ELISpot conditions. In addition, UCLA is in the process of purchasing a microscope and accompanying software for the automated reading of ELISpot plates. This will make this currently cumbersome task both quicker and more objective.

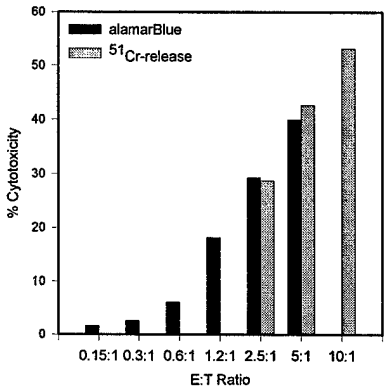

Figure 2. Alamar Blue Assay measures cytotoxicity as effectively as ^{51}Cr-release assay. LAK effector cells, Her-2 expressing tumor cell line target.

In summary, the progress in the past year is consistent with the proposal Statement of Work, and leaves us well-positioned to achieve the next goals of the proposal.

APPENDIX 1. KEY RESEARCH ACCOMPLISHMENTS

- Received IRB approval for recruitment fliers and informed consent forms
- Initiated recruitment of patients for *ex vivo* studies
- Optimized Alamar Blue assay for the measurement of tumor cytotoxicity
- Developed an improved ELISpot stimulation protocol for measuring the frequency of antigen-specific cytokine producing T cells
- Determined best method for preparing and using tumor lysate
- Synthesized and purified two HLA-A2-restricted Her-2 peptides
- Designed strategy for expression of Her-2 cDNA in DC
- Investigated additional methods for pulsing DC, including apoptotic cell bodies and whole tumor antigen

APPENDIX 2. REPORTABLE OUTCOMES

1. Promotion from Assistant Researcher to Assistant Adj. Professor is pending.

APPENDIX 3. CITED MANUSCRIPTS AND ABSTRACTS

Not Applicable: No manuscripts or abstracts during this grant period.